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Temperature effects on Zn-responses and Zn-reclamation capacity of two native Brazilian plant species: Implications of climate change

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ABSTRACT

Climate changes implications on the physiological responses and Zn-reclamation capacity of two native Brazilian plant species [*Albizia polycephala* and *Pterogyne nitens* (Fabaceae)] were studied. Plants were grown in substrate amended with 0 and 200 mg kg^{-1}, under increasing temperatures (25 °C, 30 °C and 35 °C) and plant physiological responses related to energetic and oxidative metabolism and Zn-uptake and subcellular localization were evaluated. By impairing the mitochondrial metabolism, Zn was seen to induce hydrogen peroxide (H_2O_2) production, which was stimulated by increasing temperature. However, increasing temperature was also seen to stimulate the activity of antioxidant enzymes, attenuating oxidative damage induced by H_2O_2 in leaves and roots. Moreover, increasing temperature was responsible for changes in the subcellular localization of Zn, increasing its allocation in the soluble fraction (mainly composed of vacuoles and cytoplasm) - which could be a tolerance mechanism allowing plants to survive in conditions of toxic levels of Zn under increased temperatures. Our results point to an interactive effect of rising temperature on Zn toxicity to plants. Although rising temperatures led to increased uptake of Zn and increased its deleterious effects to mitochondria metabolism (which led to increased H_2O_2 production), the increases in temperature also promoted the activity of antioxidant enzymes, responsible for H_2O_2 scavenging, thus attenuating the deleterious effects of this ROS on the cell metabolism of plants. Finally, increases in temperature do not constrain the ability of *A. polycephala* and *P. nites* to reclaim Zn and, therefore, these species should continue to be used in Zn-phytoremediation programs even given the prospects of increasing temperatures over the next century due to climatic changes.

1. Introduction

The uptake or binding of hazardous compounds by plants constitutes the base of phytoremediation, a promising technology for cleaning up contaminated environments. Several studies have been performed to identify plant species able to be used in phytoremediation programs (Cuypers et al., 2001). Moreover, the physiological mechanisms of tolerance to contaminants have been used for the selection of species and to better understand the phytoremediation capacity of plants (Gomes et al., 2013b).

Among phytoremediation methodologies, phytoextraction refers to the removal from soil and accumulation of contaminants into the harvestable parts of plants; phytostabilization, in turn, is the reduction of the mobility or bioavailability of contaminants in soil (Ali et al., 2013). Several studies have reported the tolerance of native Brazilian plant species and identified their potential use in phytoextraction or phytostabilization of trace-elements (Bicalho et al., 2017; Gomes et al., 2013a, 2012; Gomes et al., 2014a,b; Marques et al., 2000). The natural

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presence of relatively high amounts of these chemicals in Brazilian soils (Fadigas et al., 2010) may have selected physiological mechanisms in many native plant species that allow them to tolerate exposure to toxic concentrations. However, in the context of climatic changes, a new question arises: how do plants respond to the presence of hazardous compounds, such as trace-elements, under changes in temperature?

Fluctuation in natural environmental conditions are known to alter the effects of chemical stressors (Chalifour and Juneau, 2011; Folt et al., 1999; Gomes and Juneau, 2017). However, its interactive effects with toxic compounds on plant physiology have yet to be thoroughly addressed. Particularly poorly understood are the effects of temperature change on the phytoremediation capacity of plants. In Brazil, 25% increases in temperatures over the next 50 yr is expected (Siqueira and Peterson, 2003), which could limit plant performance and ability to be used for contaminant reclamation from soils.

Physiological characteristics of plants responsible for trace-element tolerance, such as photosynthesis, respiration and, mainly, the activity of antioxidant systems are also intrinsically related to temperature changes (Bicalho et al., 2017; Staehr and Sand-Jensen, 2006). The production of reactive oxygen species (ROS), whose accumulation is deleterious to plants, is induced by trace-elements. Therefore, antioxidant enzymes are of central importance in the presence of trace-elements, since they are responsible for ROS-scavenging. Moreover, the trace element phytoremediation capacity of plants has been related to the activity of enzymatic antioxidant systems, such as those of ascorbate peroxidase (APX) and catalase (CAT) (Gomes et al., 2013a). In this context, it is important to better understand the effects of changing temperatures on the phytoremediation capacity of plants used for trace-element reclamation.

Although Zn is an essential micronutrient for plant growth and development, as a component of enzymes and regulatory transcriptional proteins (Vallee and Falchuk, 1993), high concentrations of Zn become toxic, negatively affecting plants (Ebbs and Kochian, 1997). For instance, Zn can induce decreases in electron transport yields in photosystem II and in pigment biosynthesis, and cause oxidative damage via ROS generation (Chaoui et al., 1997; Cuypers et al., 2001; Tripathy and Mohanty, 1980). In seeds, the mitochondrial electron transport chain (ETC) has been proposed as the main site for Zn-induced ROS formation (Gomes et al., 2016). ROS accumulation and its related deleterious effects must be the main response of plants when submitted to Zn-toxic concentrations, since the activation of antioxidant enzymatic systems allows Zn tolerance and reclamation by plants (Gomes et al., 2013a). Therefore, as the activity of mitochondrial ETC-related and antioxidant enzymes are under temperature control, we hypothesized that the Zn-phytoremediation capacity of plants must be modulated by temperature changes.

Temperature must also modulate Zn uptake by plants since it modulates plant transpiration, which is intrinsically related to uptake and distribution of metals in the plant body. Under high temperatures, transpiration process may increase, leading to greater metal entrance in roots. By binding toxic metals into roots cells, some plants can decrease or avoid their deleterious effects in photosynthetic tissues (Gomes et al., 2011). In this context, cell walls are particularly important in limiting the flow of metals, chelating them in their largely available negative charges (Gomes et al., 2011). However, once inside cells, metals can also be stored in vacuoles, which acts as storage sites, limiting free access of toxic metals to important metabolic sites such as chloroplasts and mitochondria. In addition to modulating Zn uptake, we hypothesized that temperature may influence the organ and subcellular distribution of Zn, since it is closely related to plant transpiration and cell morphogenesis processes, such as cell wall formation and expansion (Stefanowska et al., 1999; Taiz, 1984).

In the present study, we investigated the effects of increasing temperatures (25 °C, 30 °C and 35 °C) on Zn-physiological responses and Zn-reclamation capacity of two native Brazilian plant species (*Albizia polycephala* and *Pterogyne nitens*, Fabaceae) used for trace-element phytoremediation (De Marco et al., 2016; Paiva et al., 2014). In addition to underlining the interactive effects of temperature and Zn on plant physiology, we aimed to understand how climatic changes could restrain the effective use of these species in Zn-phytoremediation programs.

2. Material and methods

2.1. Greenhouse experiments

Seedlings of *Albizia polycephala* (Benth.) Killip and *Pterogyne nitens* Tul. (Fabaceae) were acquired from the seedling nursery of the "Projeto Semeando Florestas Colhendo Águas – Núcleo Itabira" (Gouveia, Brazil). Before the Zn-treatments, plants were grown for 90 days in the nursery under greenhouse conditions (17/27.8 °C temperature min/max), in polyvinyl chloride (PVC) tubes (125 cm^3) filled with 400 g of a 2:2:1 (m/m/m) mixture of MaxFertil (15% P_2O_5, 18% Ca, 8% S; Pouso Redondo, Brazil),"Osmocote Plus 15-9-12″ (15% N, 9% P_2O_5, 12% K_2O, 1.3% Mg, 5.9% S, 0.05% Cu, 0.46% Fe, 0.06% Mn and 0.02% Mo; Dublin, USA - with nutrient release duration of six months) and sand substrates. Uniform and healthy plants were brought to the laboratory and then submitted to Zn treatments (0 and 200 mg kg^{-1}). Zn was added as $ZnSO_4.7H_2O$. The tubes were watered with 10 ml of distilled water (for control) or Zn solution, while assuring no liquid runoff from tubes. The Zn concentration used was based on concentrations encountered in Brazilian contaminated soils (Gomes et al., 2016). The substrate pH (1:1 substrate water ratio^{-1}) was adjusted to 6.7 ± 0.1 by the manual addition of 1 M HCl or 1 M NaOH (depending on the observed pH in the Zn-contaminated soil where plants were found growing). Plants were then, arranged randomly in growth chambers and held at 25 °C, 30 °C and 35 °C under a 12-h photoperiod (45 µmol m^{-2} s^{-1}, Philips T2 40 W/4 lamps). These temperatures were chosen based on the mean annual temperatures in the *Cerrado* region where the plants naturally occur, which range from 25 °C to 26 °C. Based on conservative estimates of future climate change in Brazil, a 25% increase is expected for *Cerrado* temperatures over the next 50 years (Miranda et al., 1993; Siqueira and Peterson, 2003). Plants were watered twice a day (12/12 h) with 10 ml of distilled water. A total of three replicates per treatment were used in a completely randomized experimental design.

After 25 days of treatment induction, photosynthesis (using chlorophyll fluorescence kinetic measurements) and pigment content (SPAD) were analyzed. The, plants were harvested, thoroughly washed with tap water and divided into root and shoot fractions. The samples were immediately frozen in liquid nitrogen and stored in aluminum foil paper at -20 °C until analyses.

2.2. Photosynthetic pigment and chlorophyll fluorescence analyses

Photosynthetic pigment and chlorophyll fluorescence were measured on samples from the first, second and third fully expanded leaves (second–fourth leaves from the apex), for a total of three measurements per plant. Three readings using a SPAD-502 portable chlorophyll meter (Minolta, Japan) were obtained for each leave. The leaves were then dark acclimated for 20 min and chlorophyll fluorescence emission was assessed using a pulse-amplitude modulation (PAM) fluorometer (model PAM-2500, WALZ, Effeltrich, Germany). Fluorescence results from the 76 µmol photons m^{-2} s^{-1} were used for the evaluation of the maximal photochemical efficiency of PSII (F_V/F_M) (Kitajima and Butler, 1975).

2.3. Biochemical assays

To study antioxidant enzymes, ~0.1 g of plant tissue (leaves or roots) was ground in 1000 µl of extraction solution containing 100 mM potassium buffer (pH 7.8), 100 mM EDTA, 1 mM *L*-ascorbic acid, and

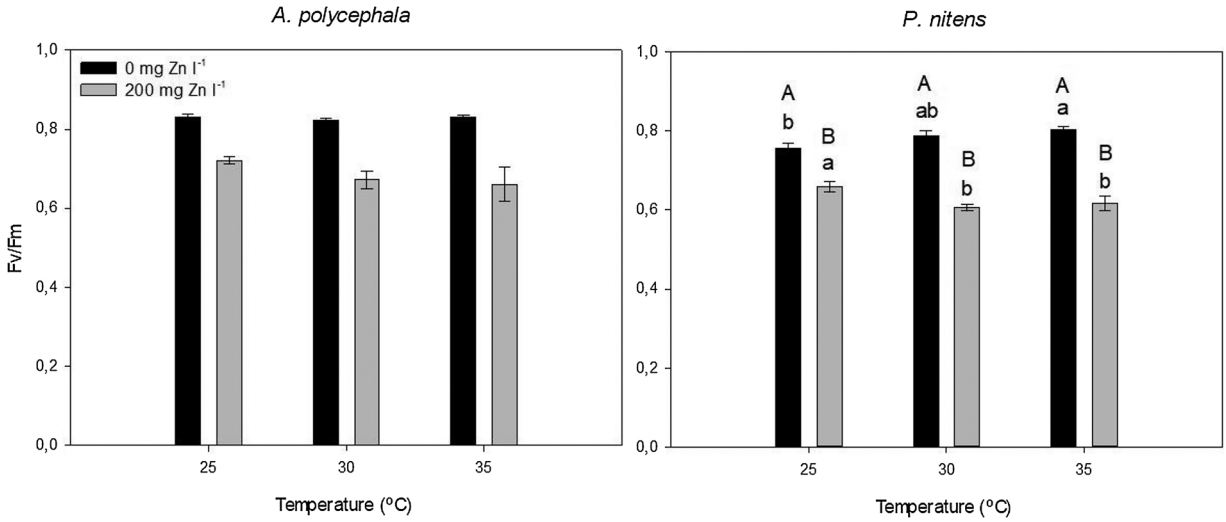

Fig. 1. Maximal photochemical efficiency of PSII (F_V/F_M) in leaves of *Albizia polycephala* and *Pterogyne nitens* plants grown in substrate amended with 0 and 200 mg l^{-1} at different temperatures (25 °C, 30 °C and 35 °C). Values expressed as means ± standard error of three replicates. Capital letters denote significant differences between Zn concentrations within the same temperature while lowercase letters denote significant differences between temperatures for the same Zn concentrations, by the post hoc Tukey test (significance at $P < 0.05$).

2% polyvinylpyrrolidone (PVP) (m/v). After determining total protein content (following the Bradford method), the activities of ascorbate peroxidase (APX; EC 1.11.1.11) (Nakano and Asada, 1981) and catalase (CAT; EC1.11.1.6) (Aebi, 1984) were measured. Hydrogen peroxide (H_2O_2) concentrations were measured following the method of Velikova et al. (2000), using 0.1 g of plant tissue.

To study the effects of Zn on mitochondrial electron transport chain (ETC) activity, the enzymatic activities of Complexes I–IV were determined spectrophotometrically in leaves, following the methods described in Huang et al. (2015). Intact mitochondria from plants were obtained following the method of Howell et al. (2006) with modifications proposed by Murcha and Whelan (2015). 50 mg of protein were used in each assay.

2.4. Chemical analyses

Total Zn and subcellular distribution of Zn within leaves and roots were determined. For total Zn concentration, 0.1 *g* of plant tissue was digested in a microwave oven (MARS-5®, CEM) at 175 °C for 10 min following USEPA Method 3051 A. After digestion, the solutions were cooled, filtered through Whatman nº 40 filter paper, and brought to a final volume of 10 ml with ultra-pure water. The filtered extracts were preserved under refrigeration (4 °C) until analysis. The subcellular distribution of Zn was determined according to (Qin et al. (2017) with modifications. ~0.3 *g* fresh tissue samples were homogenized using a mortar and pestle in 5 ml of a pre-cold extraction buffer containing 250 mmol l^{-1} sucrose, 50 mmol l^{-1} tris−HCl (pH 7.5), 1 mmol l^{-1} $MgCl_2$, and 5 mmol l^{-1} ascorbate. Firstly, the extract was centrifuged at 2050 × *g* for 10 min, and the precipitate was designated as the cell wall fraction consisting mainly of cell walls and cell wall debris. Next, the supernatant solution was re-centrifuged at 12,850 × *g* for 50 min, and the precipitate and supernatant solution were individually designated as the organelle fraction (composed of membrane and organelle components) and the soluble fraction (mainly composed of vacuoles and cytoplasm). Centrifugations were performed at 4°C. The cell wall and organelle-containing fractions were digested in a microwave oven, brought to a final volume of 10 ml with ultra-pure water, and filtered. Zinc concentrations were determined using a flame atomic absorption spectrophotometer (PerkineElmer Analyst 800, Norwalk, CT). The accuracy of the elemental analyses for Zn was confirmed by passing standard reference materials through the digestion and analytical processes. Each batch analyzed included reference material (BCR 414 Plankton, Community Bureau of Reference) with known levels of Zn, as well as one blank sample for quality control. Total Zn recovery in the reference material was approximately 85.0%.

2.5. Statistical analyses

Statistical analyses were performed using JMP 7.0 software (SAS Institute Inc.). The results were expressed as the average of three replicates. Data for each species separately were tested for normality (Shapiro–Wilk) and homoscedasticity (Bartlett) and then statistically evaluated using two-way analysis of variance (ANOVA). Interactions between Zn addition (0 and 200 mg l^{-1}) and temperature (25 °C, 30 °C and 35 °C) were included in the model. When differences were detected by ANOVA, the means were compared by the post hoc Tukey test (significance at $P < 0.05$). In the Results section, $F_{1;2}$ represents the F values for *A. polycephala* and *P. nitens*, respectively.

3. Results

3.1. Photosynthetic pigment and chlorophyll fluorescence analyses

Photosynthetic pigment, as evaluated by SPAD, was not affected by Zn concentration or temperature in both species ($P > 0.05$; data not show). The maximal photochemical efficiency of PSII (F_V/F_M) in both species was decreased by the addition of Zn ($P < 0.0001$; Fig. 1). No significant interaction ($P > 0.05$) between Zn concentration and temperature was observed for *A. polycephala* ($F = 3.28$), while it was significant ($P < 0.0001$) for *P. nitens* ($F = 23.51$). In *P. nitens* without Zn treatment, F_V/F_M was greater in plants kept under 35 °C than in those under 25 °C. In contrast, when treated with 200 mg l^{-1}, F_V/F_M was greater in plants kept under 25 °C than in those under 30 °C and 35 °C (Fig. 1).

3.2. Hydrogen peroxide

Regardless of the temperature, H_2O_2 concentrations increased significantly ($P < 0.001$) in leaves ($F_{1;2} = 45.17;13.82$) of plants submitted to Zn treatment. A significant interaction ($P < 0.0001$) between Zn concentration and temperature was observed for H_2O_2 concentration in leaves ($F_{1;2} = 8.14; 7.66$; $P < 0.001$; Fig. 2). In *A. polycephala* plants treated with 200 mg l^{-1} and in *P. nitens* without Zn-treatment, H_2O_2 concentration was lowest in leaves of plants kept under 25 °C (Fig. 2).

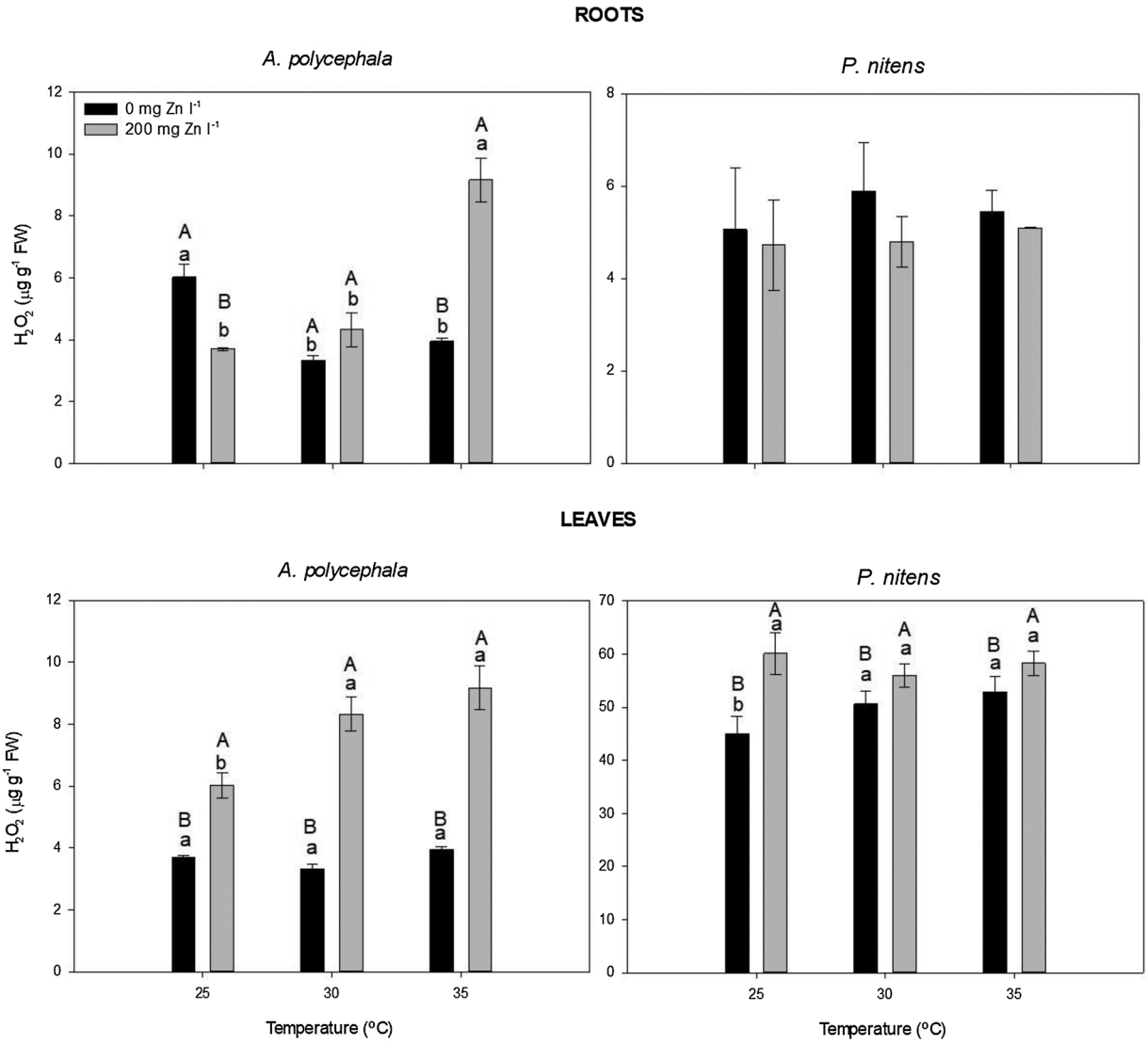

Fig. 2. Hydrogen peroxide (H_2O_2) in leaves and roots of *Albizia polycephala* and *Pterogyne nitens* plants grown in substrate amended with 0 and 200 mg l^{-1} at different temperatures (25 °C, 30 °C and 35 °C). Values expressed as means ± standard error of three replicates. Capital letters denote significant differences between Zn concentrations within the same temperature while lowercase letters denote significant differences between temperatures for the same Zn concentrations, by the post hoc Tukey test (significance at $P < 0.05$).

H_2O_2 concentrations in roots ($F = 45.17$) increased significantly ($P < 0.001$) in *A. polycephala* plants treated with 200 mg l^{-1}. A significant interaction ($P < 0.0001$) between Zn concentration and temperature was observed for H_2O_2 concentration in roots of *A. polycephala* ($F = 128.07$). In plants without Zn treatment, H_2O_2 concentration was highest in roots of plants kept under 25 °C. In contrast, in plants treated with 200 mg l^{-1}, the highest H_2O_2 concentration was observed in plants kept under 35 °C (Fig. 2). In *P. nitens*, H_2O_2 concentrations in roots were not significantly affected ($P < 0.05$; Fig. 2).

3.3. Antioxidant enzymes

Fig. 3 shows the activity of antioxidant enzymes (APX and CAT) in leaves of the studied plants. Ascorbate peroxidase ($F_{1;2} = 160.42$; 28.52) and catalase ($F_{1;2} = 53.40$; 64.01) activity in leaves increased significantly ($P < 0.001$) in plants of both species treated with 200 mg l^{-1}. Moreover, APX ($F_{1;2} = 39.74$; 53.87) and CAT ($F_{1;2} = 46.06$; 42.83) activity in leaves increased ($P < 0.001$) as the temperature increased. For *A. polycephala*, a significant interaction between Zn concentration and temperature was observed for APX ($F = 11.03$; $P < 0.001$) and CAT ($F = 4.94$; $P < 0.05$) activity in leaves. In plants treated with 200 mg l^{-1}, APX activity in leaves increased as temperature increased (Fig. 3). With respect to CAT, activity was lower in plants treated with 200 mg l^{-1} kept under 25 °C than in those under 30 °C and 35 °C; in plants without Zn treatment (0 mg l^{-1}), CAT activity of leaves increased as temperature increased (Fig. 3).

The activity of APX and CAT in roots of the studied plants is presented in Fig. 4. Similar responses of APX and CAT activity in roots were observed for both species. The treatment with 200 mg l^{-1} significantly increased ($P < 0.001$) APX ($F_{1;2} = 171.77$; 350.11) and CAT ($F_{1;2} = 80.03$; 393.39) activity in roots. Moreover, APX ($F_{1;2} = 9.26$; 18.18) and CAT ($F_{1;2} = 7.22$; 36.29) activity in roots increased ($P < 0.001$) as temperature increased. A significant interaction ($P < 0.01$) between Zn concentration and temperature was observed for APX ($F_{1;2} = 9.26$; 18.18) and CAT ($F_{1;2} = 4.46$; 5.60) activity in roots of both species. In *A. polycephala* treated with 200 mg l^{-1}, APX and CAT activity was greater in roots of plants kept under 35 °C. Similarly, in *P. nitens* treated with 200 mg l^{-1}, APX activity was greater in roots of plants kept under 35 °C, however, CAT activity in roots did not differ significantly between plants kept under 30 °C and 35 °C; in plants

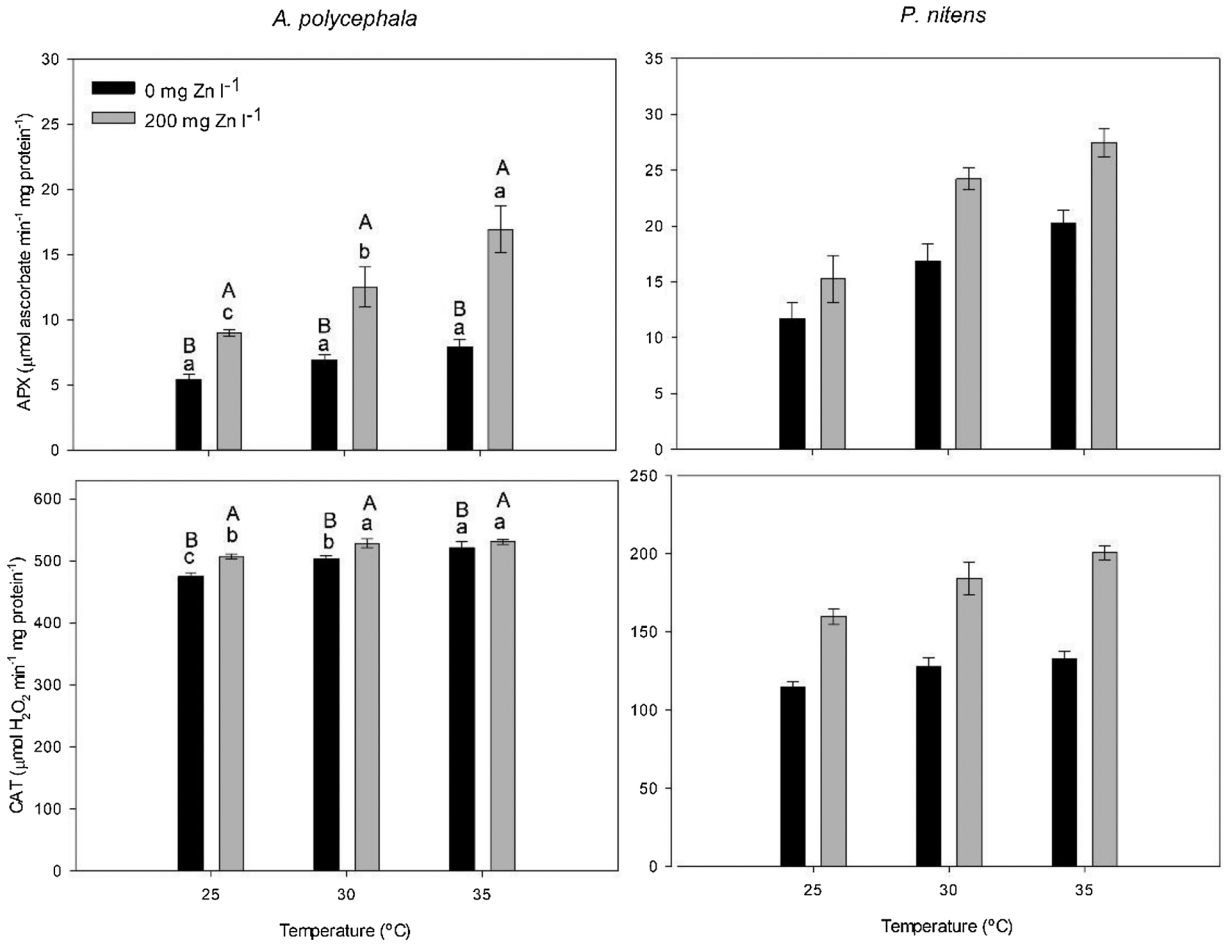

Fig. 3. The activities of ascorbate peroxidase (APX) and catalase (CAT) in leaves of *Albizia polycephala* and *Pterogyne nitens* plants grown in substrate amended with 0 and 200 mg l^{-1} at different temperatures (25 °C, 30 °C and 35 °C). Values expressed as means ± standard error of three replicates. Capital letters denote significant differences between Zn concentrations within the same temperature while lowercase letters denote significant differences between temperatures for the same Zn concentrations, by the post hoc Tukey test (significance at $P < 0.05$).

without Zn treatment, CAT activity was greater in roots of plants kept under 35 °C than in roots of those kept under 25 °C.

3.4. Mitochondrial electron transport chain enzymes

In both species, the activity of all mitochondrial electron transport chain enzymes in leaves (Complex I-IV) decreased significantly ($P < 0.001$) with Zn treatment and increased as temperature increased (Fig. 5; Table 1). For *P. nitens*, a significant interaction between Zn concentration and temperature was observed for Complex II and IV activity in leaves. In plants without Zn-treatment, the activity of these enzymes increased as temperature increased. Similarly, the activity of Complex IV in leaves of plants treated with Zn increased as temperature increased (Fig. 5).

3.5. Zn concentrations and distribution

Regardless of the temperature, Zn concentration in roots of both species increased in plants treated with 200 mg l^{-1} ($P < 0.001$; Fig. 6). A significant interaction ($P < 0.05$) between Zn concentration and temperature was observed for Zn concentration in roots ($F_{1;2} = 8.33$; 4.81). In both species, Zn concentration in roots of plants treated with 200 mg l^{-1} increased as temperature increased (Fig. 6). In leaves, Zn concentration increased significantly ($P < 0.001$) in plants of the Zn treatment ($F_{1;2} = 8.33$; 4.81) and was lower ($P < 0.001$) in plants kept under 25 °C (Fig. 6).

The subcellular distribution of Zn in roots and leaves was similar for both species. Zn addition reduced Zn concentration in the cell wall fraction. Moreover, in plants treated with Zn, rising temperatures and Zn addition resulted in decreased concentration of Zn in the cell wall fraction in roots and leaves while it increased in the soluble and organelle fractions ($P < 0.05$; Fig. 7).

4. Discussion

It is not surprising that high amounts of Zn induce deleterious effects in plants (Ebbs and Kochian, 1997; Gomes et al., 2013a; Tripathy and Mohanty, 1980). However, our results point to the modulation of Zn-toxicity by temperature in two plant species used in metal-phytoremediation programs in Brazil. This means that changes in temperature due to climate change will alter the ability of plants to survive under conditions of toxic levels of Zn, with effects on their phytoremediation capacity. According to conservative estimates, regions of occurrence of *A. polycephala* and *P. nitens* (or areas in which the species are used in recovering programs) will experience a 25% increase in temperature over the next 50 years (Siqueira and Peterson, 2003). Therefore, it is important to understand how these species will respond to rising temperatures under a situation of increased levels of Zn.

In both studied species, the addition of 200 mg l^{-1} to the growth substrate of plants resulted in a significant decrease in the maximal photochemical efficiency of PSII (F_V/F_M; Fig. 1). Since F_V/F_M is a proxy of PSII integrity (Walter et al., 2003), decreased F_V/F_M in Zn-treated plants indicates injuries to the photosynthetic apparatus (Gomes et al., 2017a,b). Although photosynthetic pigments (as evaluated by SPAD)

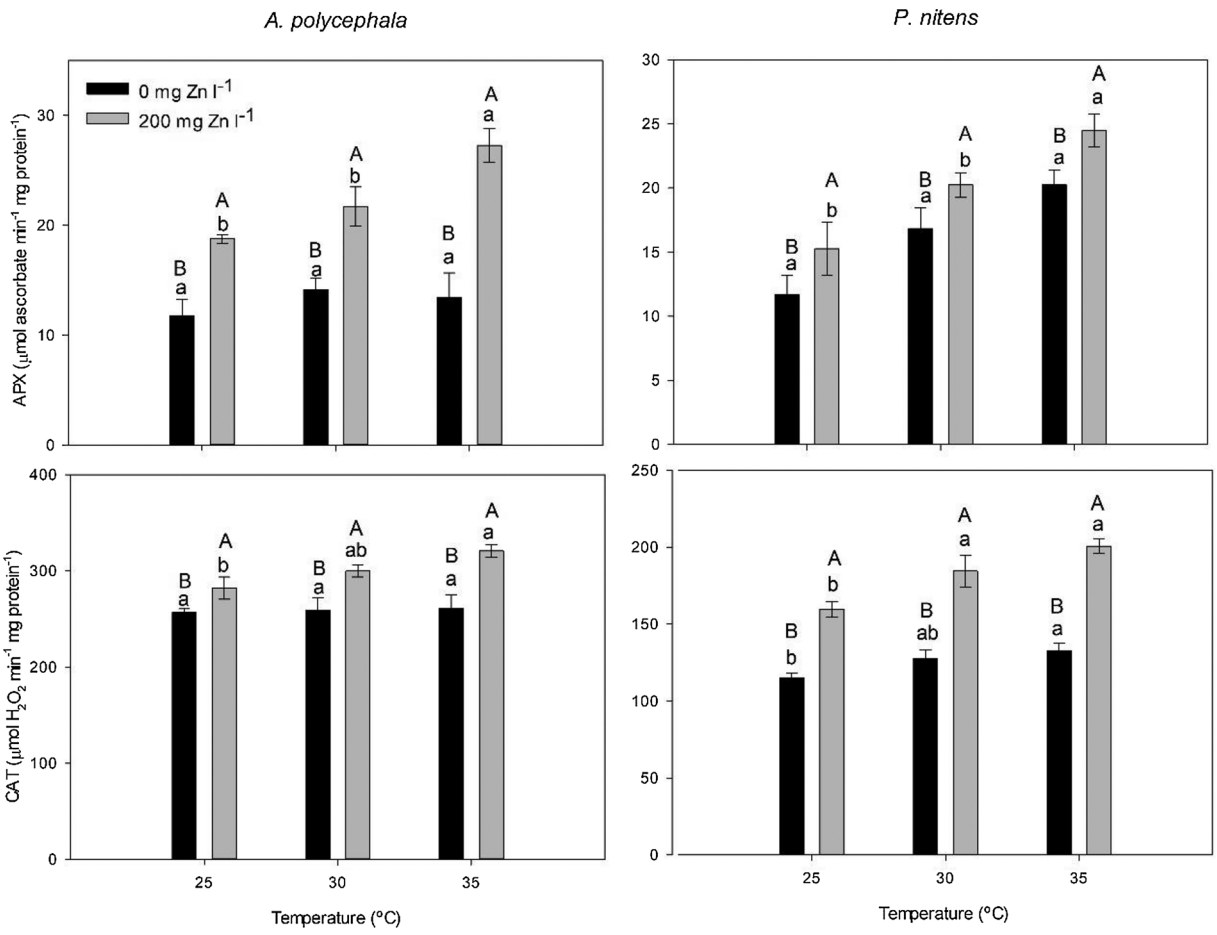

Fig. 4. The activities of ascorbate peroxidase (APX) and catalase (CAT) in roots of *Albizia polycephala* and *Pterogyne nitens* plants grown in substrate amended with 0 and 200 mg l^{-1} at different temperatures (25 °C, 30 °C and 35 °C). Values expressed as means ± standard error of three replicates. Capital letters denote significant differences between Zn concentrations within the same temperature while lowercase letters denote significant differences between temperatures for the same Zn concentrations, by the post hoc Tukey test (significance at $P < 0.05$).

did not change in Zn-treated plants, their PSII structure (such as structural proteins) must have been affected by the metal. Interestingly, we observed a relationship between the F_V/F_M and H_2O_2 concentrations in the leaves of the plants. Plants showing increased H_2O_2 concentrations in their leaves also showed decreased F_V/F_M (Fig. 1 and 2). PSII proteins are quite sensitive to H_2O_2, which can, for instance, suppress the *de novo* synthesis of D1 protein - a constitutive protein required for PSII repair and assembly (Takahashi and Murata, 2008). The disruption of photochemistry (due to PSII-damage) in Zn-treated plants invariably contributes to ROS production due to the leak of electrons to oxygen molecules in chloroplasts. However, we also observed that Zn treatment induced changes in the activity of mitochondria electron transport chain enzymes – which is one of the main sources of ROS for cells.

Zn was proposed to impair ubiquinone reduction, which leads to ROS formation in Complex I and II in embryos of *Dimorphandra wilsonii* seeds (Gomes et al., 2016). Similarly, we also observed decreased activity of Complex I and II, and to a greater extent than in the other evaluated complexes, in leaves of plants treated with Zn (Fig. 5). Although Zn has been proposed to impair photosystem II functioning by modifying the site of the secondary quinone acceptor of PSII (Mohanty et al., 1989), as discussed before, our data attest to damage to PSII structure, which can be related to increased H_2O_2 concentration in leaves upon Zn-exposure. Therefore, we can argue that by disrupting the activities of Complex I and II in mitochondria, Zn accentuates ROS production, which will further affect thylakoid structure and photosynthesis (Richter, 1992). By using laser scanning confocal microscopy, Gomes and Juneau (2016) showed that initial ROS formation in *Lemna minor* plants exposed to glyphosate occurs outside the chloroplasts, but the final result of ROS accumulation is their entrance into chloroplasts and the disruption of photosynthesis due to ROS interference with PSII structure. A similar process must occur in leaves of Zn-treated plants, with ROS produced in mitochondria further affecting the photosynthetic apparatus.

Having identified the possible mechanism of action of Zn in plants, it is important to discuss the integrative effects of rising temperatures on the physiology of plants exposed to the metal. Temperature modulates the activity of enzymatic complexes in the mitochondrial apparatus (Patanè et al., 2006) and rising temperatures stimulate respiratory activity in plants (Bicalho et al., 2017). Indeed, in plants without Zn treatment, we observed increased activity of mitochondria electron transport chain enzymes in leaves of plants kept under 30 °C and 35 °C in relation to those kept under 25 °C (Fig. 5). However, if the mitochondrial electron transport chain is a Zn target, the stimulation of the respiratory activity by temperature can accentuate ROS formation in mitochondria of plants exposed to the metal. This was the case observed in *A. polycephala* leaves, in which the rise in temperature led to increased H_2O_2 concentrations in plants treated with Zn. However, this was not seen in *P. nitens* plants exposed to the metal. We were, therefore, interested in the activity of antioxidant enzymes in leaves to better understand the effects of temperature on Zn-induced ROS formation.

Enzymes such as ascorbate peroxidase (APX) and catalase (CAT) are responsible for the scavenging of H_2O_2 and are quite responsive to environmental stresses (Foyer and Noctor, 2011; Gomes et al., 2017a,b). Under situations of ROS accumulation, such as driven by

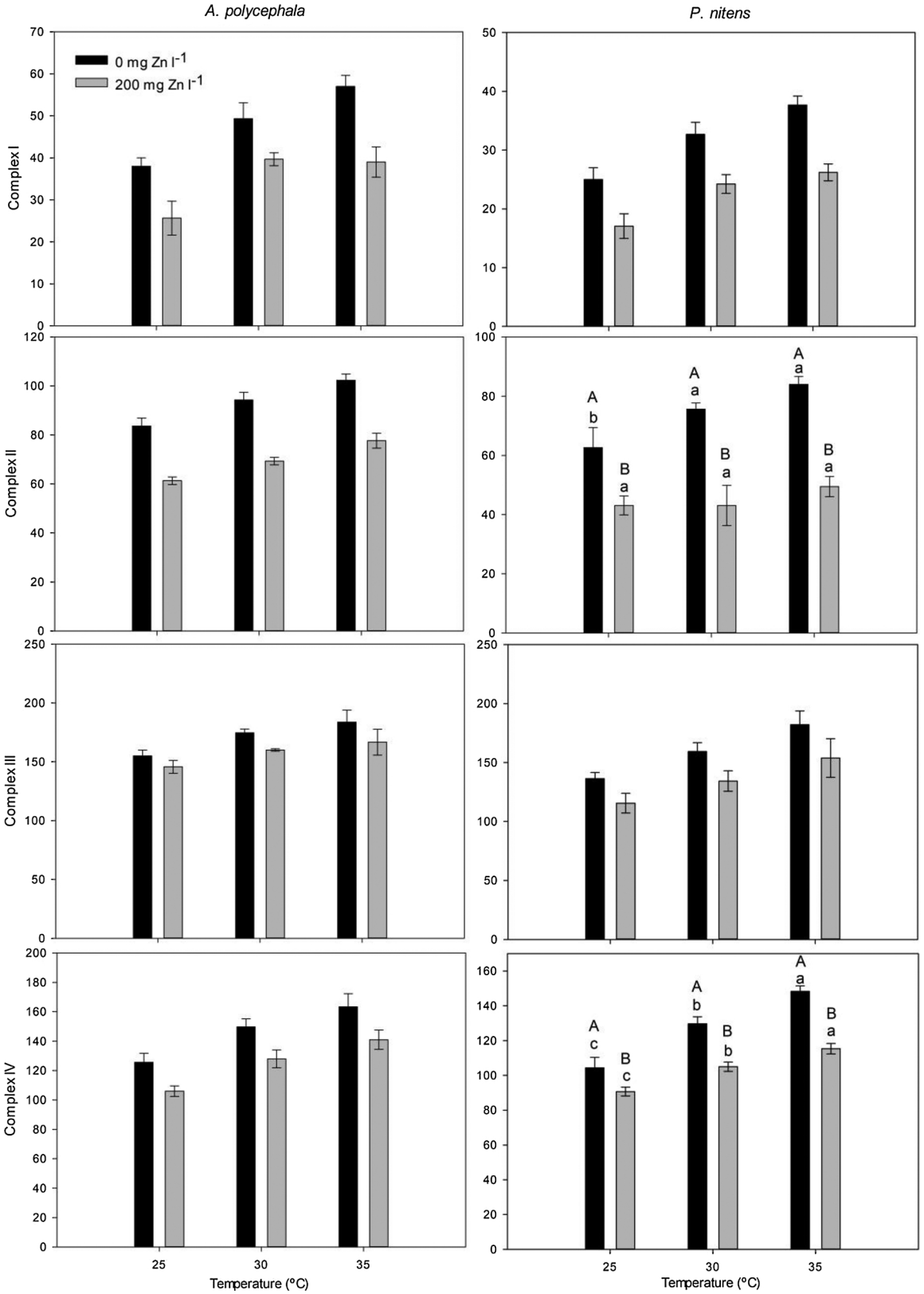

Fig. 5. Complex I (NADH:ubiquinone oxidoreductase; μmol NADH mg protein^{-1} s^{-1}), Complex II (Succinate dehydrogenase; μmol NADH mg protein^{-1} s^{-1}), Complex III (Ubiquinol-cytochrome c reductase; μmol cytochrome cIII mg protein^{-1} s^{-1}) and Complex IV (Cytochrome c oxidase; μmol cytochrome cIII mg protein^{-1} s^{-1}) activities in leaves of *Albizia polycephala* and *Pterogyne nitens* plants grown in substrate amended with 0 and 200 mg l^{-1} at different temperatures (25 °C, 30 °C and 35 °C). Values expressed as means ± standard error of three replicates. Capital letters denote significant differences between Zn concentrations within the same temperature while lowercase letters denote significant differences between temperatures for the same Zn concentrations, by the post hoc Tukey test (significance at $P < 0.05$).

Table 1
Two-way analysis of variance (ANOVA) for the effects of Zn addition (Zn) and temperature (ºC) on various measures[a] in *Albizia polycephala* and *Pterogyne nitens* plants.

Source of variation	*polycephala*				*P. nitens*			
	CI	CII	CIII	CIV	CI	CII	CIII	CIV
Zn	84.2***	388.8***	17.2***	50.4***	118.2***	179.2***	25.9***	180.2***
ºC	45.6***	69.0***	19.8***	50.4***	59.9***	13.7***	24.9***	126.3***
Zn x ºC	2.8	0.4	0.4	0.1	1.6	4.7*	0.1	9.9**

*, ** and *** are significant *p* values at 0.1, 0.01 and 0.001, respectively.
[a] Zinc in mg l^{-1} and temperatures (25 °C, 30 °C, and 35 °C). Measures were Complex I (NADH:ubiquinone oxidoreductase), Complex II (Succinate dehydrogenase), Complex III (Ubiquinol-cytochrome c reductase) and Complex IV (Cytochrome c oxidase).

metal exposure, the antioxidant system is often activated (Feng et al., 2016) to prevent the hazardous effects of ROS to cell structures such as proteins, lipids and nucleic acids. Antioxidant enzymes are also sensitive to temperature changes, increasing their activity under rising temperatures (Bicalho et al., 2017). While in both species plants treated with Zn showed greater APX and CAT activity in leaves under higher temperatures (30 °C and 35 °C), their responses differed between the enzymes evaluated (Fig. 3). The increase in APX activity in leaves of plants kept under 30 °C and 35 °C in relation to those under 25 °C where similar between the species, ranging from 39 to 88% in *A. polycephala* and from 58 to 79% in *P. nitens*. However, the increase in CAT activity differed widely between the species, being 4–5% in *A. polycephala* while 58–79% in *P. nitens*. This significant difference in CAT activity between the species must have assured greater H_2O_2 scavenging in leaves of *P. nitens* plants submitted to Zn under 30 °C and 35 °C, which reach similar H_2O_2 levels as those under 25 °C, justifying, therefore, no significant difference in H_2O_2 concentrations in their leaves. In this context, the activity of CAT must be central in situations of plant exposure to Zn under rising temperatures.

The importance of CAT to plant tissue exposed to Zn was also noticed in roots. Under rising temperatures, the increase in CAT activity in roots of *P. nitens* was greater in relation to that of APX, and in those plants, no significant ROS accumulation was observed in roots in relation to untreated plants (Fig. 2 and 4). Moreover, in plants kept under 35 °C, the activity of CAT in plants treated with Zn greatly increased in *P. nitens* (51%) relative to *A. polycephala* (22%), in which H_2O_2 accumulation was observed. Finally, in roots of *P. nitens* plants, CAT was also highly responsive to rising temperatures, with increases ranging from 15 to 25% in plants kept under 30 °C and 35 °C, relative to those kept under 25 °C, while in *A. polycephala* the increase in CAT activity

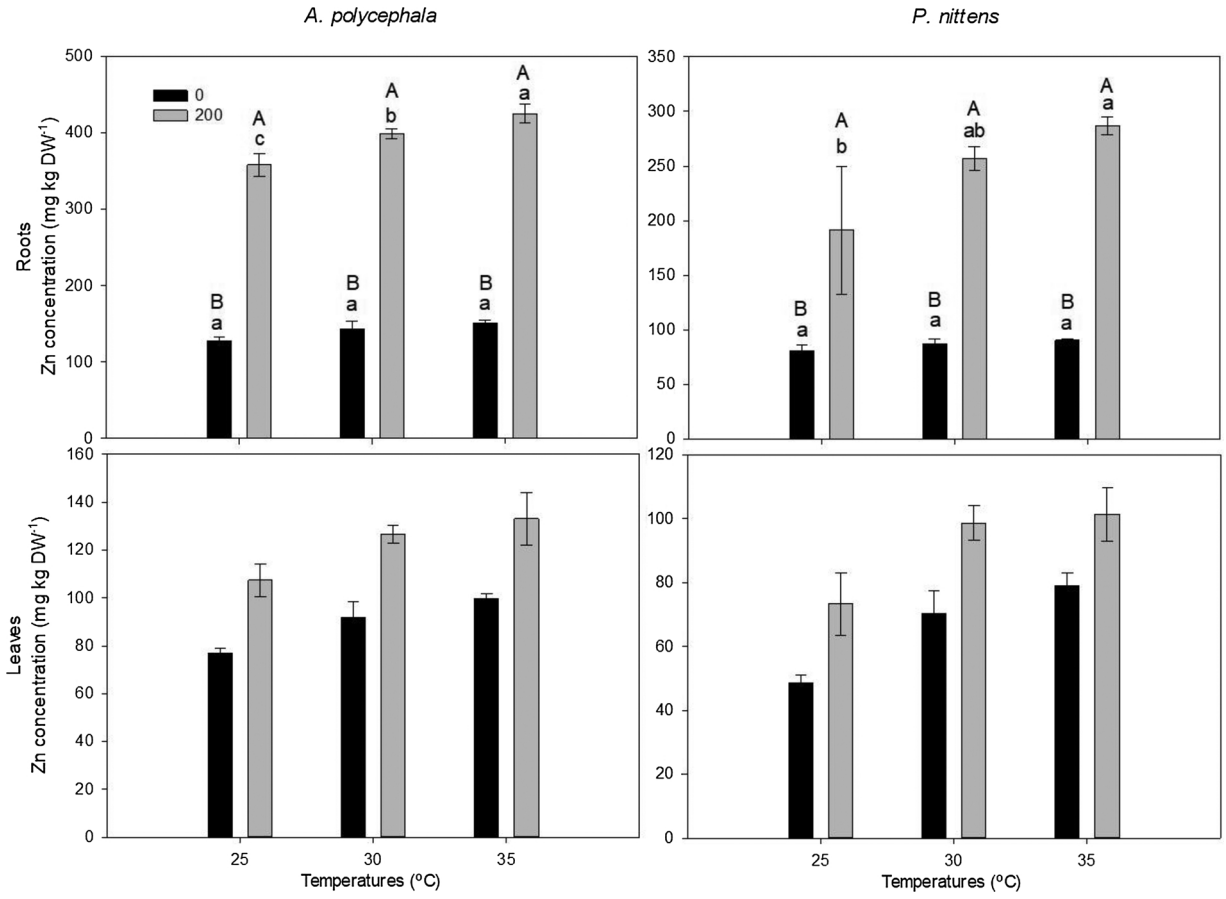

Fig. 6. Total Zn concentration in roots and leaves of *Albizia polycephala* and *Pterogyne nitens* plants grown in substrate amended with 0 and 200 mg l^{-1} at different temperatures (25 °C, 30 °C and 35 °C). Values expressed as means ± standard error of three replicates. Capital letters denote significant differences between Zn concentrations within the same temperature while lowercase letters denote significant differences between temperatures for the same Zn concentrations, by the post hoc Tukey test (significance at $P < 0.05$).

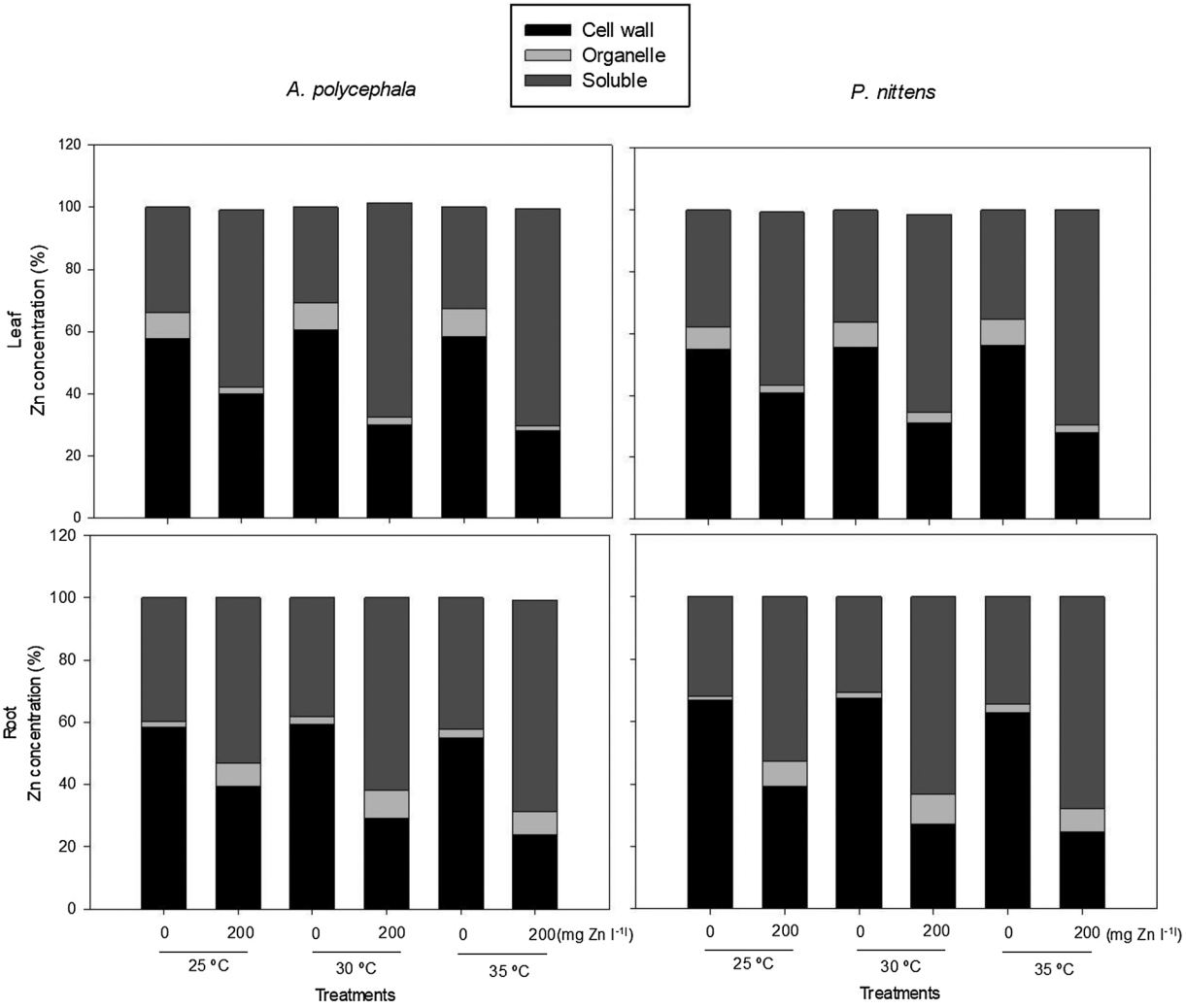

Fig. 7. Subcellular distribution of Zn in leaves and roots of *Albizia polycephala* and *Pterogyne nitens* plants grown in substrate amended with 0 and 200 mg l^{-1} at different temperatures (25 °C, 30 °C and 35 °C). Values expressed as means ± standard error of three replicates. Capital letters denote significant differences between Zn concentrations within the same temperature while lowercase letters denote significant differences between temperatures for the same Zn concentrations, by the post hoc Tukey test (significance at $P < 0.05$).

only ranged from 6 to 13%.

As expected, upon Zn exposure, the metal concentration in roots and leaves of both plant species increased (Fig. 6). Interestingly, the rise in temperature significantly increased metal concentration in roots and leaves of plants treated with Zn. Metal acquisition by plants from the soil solution is a process intrinsically related to leaf transpiration rates. Greater transpiration rates are expected under rising temperatures, which, therefore, must lead to greater absorption of available metals from the soil solution. One can argue, however, that in this case, the increase of Zn in leaves of plants submitted to the metal under rising temperatures must be greater than that observed for roots – which was not observed. In *A. polycephala* an increase of 11–18% was observed in roots and of 18–23% in leaves; in *P. nitens,* in turn, the increase in Zn concentration in roots of plants kept under 30 °C and 35 °C was of 34–50% and in leaves of 34–38% (in relation to treated plants kept under 25 °C). It is important to note, however, that several plant species used for metal phytoremediation restrict the translocation of metal from roots to shoot as a strategy to avoid metal-interference with the photosynthetic apparatus (Gomes et al., 2014a,b). In these plants, it appears that upon the acquisition of toxic amounts of metals by leaves, transpiration is restricted, reducing metal translocation and therefore its hazardous damage to green tissues (Gomes et al., 2014a,b). Moreover, metal-tolerant plants employ several morphoanatomical changes to reduce the translocation of metals to aerial parts, such as the development of thicker cell walls in roots that can adsorb and thus retain metals (Gomes et al., 2011; Lux et al., 2004). We were, therefore, interested in understanding the effects of rising temperatures in the subcellular distribution of Zn in roots and leaves of plants.

Due to the negative charges present in its structure, cell walls are the first place involved in the binding of metals (Ghosh and Singh, 2005; Hall, 2002). Indeed, in plants without Zn treatment, Zn was mainly found in cell walls (Fig. 7). The proteins and polysaccharides of cell walls have great potential of acting as ligands for binding metals, and can act to store essential nutrients such as Zn (Hayens, 1980). However, upon Zn exposure, Zn was preferably observed in the soluble fraction (mainly composed of vacuoles and cytoplasm). These results suggest that vacuoles may be the main storage sites for Zn in situations of plant exposure to an excess of the metal. Similar results were observed for the Zn-hyper-accumulator *Sedum alfreddi* (Li et al., 2006). The vacuole is a dynamic organelle and can comprise up to 90% of the total volume of some cells. Similar to cell walls, this organelle possesses organic ligands, such as amino acids and organic acids, which decreases activity of free ions, reducing their toxicity (Cosio et al., 2004). Interestingly, we were surprised to note that changes in temperature drove changes in the subcellular distribution of Zn in plants, reducing the metal concentration in cell walls in leaves and roots of plants treated

with Zn, resulting in greater allocation of Zn to the soluble fraction of cells (Fig. 7). Temperature is known to affect cell wall thickness and biosynthesis (Stefanowska et al., 1999; Taiz, 1984). High temperature impairs hemicellulose and cellulose synthesis (Gall et al., 2015) and alters cell-wall related gene expression (Yang et al., 2006). In this context, a decrease in the cell wall area available for metal retention could be associated with the decreased Zn concentrations in the cell wall observed in plants submitted to Zn treatment under rising temperatures. Moreover, increases in temperature could result in changes in the synthesis of organic ligands, including phytochelatins, which can bind metals and prevent them from circulating in the cytosol (Petraglia et al., 2014) - justifying the increased concentration of Zn in the soluble fraction of cells. However, further studies are necessary to better understand the changes in Zn-subcellular localization in response to changing temperatures.

5. Conclusion

Several native Brazilian plant species have been certified for their potential use in metal reclamation. However, little is still understood about the responses of these plants to changes in temperature under situations of metal contamination of soils. Our results point to an interactive effect of rising temperature on Zn toxicity to plants. However, both studied species showed great tolerance to the presence of an excess of Zn in their growth substrate even under increased temperatures. Although rising temperatures led to increased uptake of Zn and increased its deleterious effects to mitochondria metabolism (which led to increased H_2O_2 production), the increases in temperature also promoted the activity of antioxidant enzymes, responsible for H_2O_2 scavenging, thus attenuating the deleterious effects of this ROS on the cell metabolism of plants. Moreover, rising temperature was also responsible for changes in the subcellular localization of Zn, which could be a tolerance mechanism, allowing plants to survive in conditions of toxic levels of Zn under increased temperatures. Finally, the predicted increase in temperature (Miranda et al., 1993; Siqueira and Peterson, 2003) over the next century will not constrain the ability of *A. polycephala* and *P. nitens* to reclaim Zn, and therefore, these species should continue to be used in Zn-phytoremediation programs.

Author statement

M.P.G, D.M.D.H and Q.S.G designed the experiments, gave technical support and conceptual advice. M.P.G, J.C.M.B., J.G.S., F.V.S.C and E.M.B. performed experiments and analyzed data; M.P.G wrote the paper.

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